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Effects of Density and of Dehydration of Sickle Cells on Their Adhesion to Cultured Endothelial Cells

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Abnormal adhesion of sickle cells to vascular endothelium may be a factor in the initiation of painful vaso-occlusive crisis. The sickle cell population contains an unusually large number of less dense reticulocytes that are known to be more adhesive than mature red cells, but there is contradictory evidence regarding the adhesiveness of dense sickle cells. We used a flow-based assay of adhesion to cultured human umbilical vein endothelial cells to test the properties of density fractions of sickle cells, prepared either by density gradient or by centrifugation of packed cells. We also examined the effects of incubating sickle cells with or without cyclical deoxygenation on their adhesion. After fractionation on a Percoll-Isopaque gradient, the less dense 10% (reticulocyte-rich) cells and the most dense 10% cells adhered in greater number than the remainder (by about twofold). However, after centrifugation of packed cells, the less dense 10% were again more adhesive than the "middle" cells, but the most dense were not. Exposing sickle cells to constituents of the gradient had no consistent effect on adhesion, while centrifugal packing induced a degree of hemolysis, and tended to reduce adhesiveness of the dense fraction previously obtained from a gradient. Incubation in air at 37°C for 15 hr reduced the number of reticulocytes and the adhesiveness of less dense sickle cells compared to those held at 4°C. On the other hand, incubation at 37°C for 15 hr with cyclical deoxygenation caused formation of dense cells and increased adhesiveness compared to incubation without cyclical deoxygenation. We conclude that young, less dense sickle cells are unusually adhesive, but that this adhesiveness is reduced during maturation. However, repeated sickling in vivo causes formation of an abnormally dense subpopulation of cells which either redevelop an increased tendency to adhere to endothelial cells or preserve their initial adhesiveness. Both adhesive cell populations may be implicated in promoting vascular obstruction. © 1996 Wiley-Liss, Inc.

Key words: sickle cell anemia, endothelium, adhesion, erythrocytes

INTRODUCTION

Sickle cell disease is characterized by a chronic hemolytic anemia and repeated episodes of painful vascular-occlusive crisis linked with polymerization of deoxygenated hemoglobin S (Hb S) and consequent red cell sickling [1]. The red cell population in those homozygous for HbS (HbSS) is heterogeneous, with a high proportion of reticulocytes and young cells, as well as a variable number of unusually dense cells, including irreversibly sickled cells (ISC) [2]. These dense cells are thought to play an important part in blocking small blood vessels prior to vascular-occlusion, because of both their poor deformability when oxygenated and their increased tendency to sickle [3]. However, the variation in the percentage of dense and irreversibly sickled cells does not appear to correlate with the clinical severity of the disease on a patient-by-patient basis [4]. Other factors may therefore contribute to initiation of vascular-occlusive crisis, including an increased propensity for sickle cells to adhere to vascular endothelium [5].

Sickle cells have been shown by several workers to be more adhesive to cultured endothelial cells than normal HbAA cells in both static assays [6,7] and assays using controlled flow [8,9]. The degree of adhesiveness has been correlated with clinical symptoms of disease on a

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patient-by-patient basis [10,11], and the concept has arisen that adhesion to vascular endothelium could be an initiating factor for occlusion. Observations in in vivo and ex vivo microcirculation models support this hypothesis [12,13]. It is not clear, however, which sickle cells among the population are more adhesive, with conflicting results from different studies. It has been shown that HbAA cells with a high proportion of reticulocytes show greater adhesion than HbAA cells with a normal reticulocyte count and that adhesiveness is particularly elevated for less-dense sickle cells [8,14]. Observations in an ex vivo circulatory model indicated that not only were less dense cells more adhesive in venules than dense cells, but that dehydration per se reduced adhesiveness of the less-dense cells [15]. Thus, abnormal adhesion of sickle cells may arise, in part at least, from the high concentration of reticulocytes and young cells in the population. Recent studies have revealed a series of potential adhesion molecules on immature sickle cells that may aid binding to vascular endothelium: CD36, GpI_b-like glycoprotein and VLA-4 [14,16,17]. The first two may promote endothelial attachment via the plasma proteins thrombospondin (TSP) and von Willebrand factor (vWF), implicated in increased adhesiveness of sickle cells [14,16,18], while VLA-4 is a counter-receptor for vascular cell adhesion molecule-1 expressed on cytokine-activated endothelial cells [17]. On the other hand, early studies showed that dense cells [6], or dense cells and young cells [11], were particularly adherent, and dense sickle cells have also been shown to bind preferentially to endothelial cells stimulated with tumor necrosis factor (TNF) [19].

There may be several confounding factors when attempting to compare previous studies of sickle cell fractions. Plasma proteins have been shown to increase endothelial binding of sickle cells [20] with the effect being greater for microvascular endothelium compared to the large vessel endothelial cells more typically used in vitro [21]. As noted above, the plasma proteins TSP and vWF are particularly implicated in some mechanisms of binding of reticulocytes [16,18], and absence of plasma in some studies [6,11] could explain failure to detect high levels of binding for less-dense cells. In addition, some studies have been carried out under static conditions [6,11,16,19], while others incorporated flow [8,14,17]. Adhesion will inevitably occur in the presence of flow in vivo, and the rate of flow and the stress applied to newly formed adhesive bonds will affect the probability of stable cellular attachment. Judging from analogous studies of leukocyte adhesion, bonds adequate for attachment under stationary conditions need not necessarily operate successfully for moving cells [22]. In addition, different methods of density fractionation of red cells, e.g., isopyknic density gradient [6,11,16,19] versus high-speed centrifugation of packed cells [8,14], might yield different results depending on the resolution of the fractionation procedure and the physicochemical conditions to which the cells are exposed. Therefore, we have used a flow-based assay to investigate the adhesive properties of sickle cells fractionated by two different methods and of a dense population of cells prepared from an initially less dense reticulocyte-rich fraction by cyclical deoxygenation and reoxygenation in vitro [23,24]. In this way, we aimed to assess the effect of in vivo and in vitro aging and dehydration on adhesion of sickle cells to cultured endothelial cells.

METHODS

Preparation of Red Blood Cells

Venous blood was drawn from adults with homozygous sickle cell disease in the steady state or from healthy, adult HbAA volunteers, anticoagulated with heparin (5 IU/ml) and used on the day of withdrawal. Informed consent was obtained from all patients, none of whom had been transfused in the previous 3 months and none of whom was receiving treatment at venesection. Leukocytes and platelets were removed by filtering the blood through Imugard IG 500 cotton wool (Terumo Corporation, Tokyo, Japan), and red cells were washed twice with 40 mM HEPES buffered saline (HBS) (pH 7.4 at 25°C).

Density fractions of sickle cells were prepared in two ways: (1) by centrifugation at 24,000*g* on a Percoll-Isopaque continuous density gradient for 40 min as previously described [23]; (2) by centrifuging packed red cells (obtained from washed cells by centrifugation at 645*g* for 1 min) in disposable glass Wintrobe (erythrocyte sedimentation rate) tubes at 2,000*g* for 30 min. The Wintrobe tubes were cut with a diamond pencil to obtain the fractions required. The gradients and columns of cells were separated into the least and most dense 10% and the middle 80%, and the fractions were characterized by the percentage of reticulocytes and ISC present. The MCV and MCHC were derived from the microhematocrit (15,000*g* for 15 min) and the red cell count and hemoglobin measured by a Coulter counter (Coulter JT4; Coulter Electronics, Luton, UK).

Dense, dehydrated sickle cells were prepared in vitro by cyclical deoxygenation–reoxygenation in a cyclical gas exchanger [23]. After centrifuging unfractionated cells at 24,000*g* on a Percoll-Isopaque density gradient as above, the 50% most dense cells were discarded. The remaining less-dense fraction was suspended at 0.3×10^{12}/L in HBS (pH 7.4 at 37°C) containing KCl (5 mM), glucose (10 mM), $CaCl_2$ (2 mM) and gentamicin sulphate (32 μg/ml). The suspensions were divided into 5-ml aliquots, which were then incubated at 37°C for 15 hr while being subjected to 15-min cycles of alternating gassing with nitrogen or air to give a PO_2 varying between 5 kPa (deoxygenated–approximately venous level) and 16 kPa (reoxygenated–arterial level), as previously described

[23]. After incubation, the cells were fully reoxygenated by washing 3 times with HBS. This procedure yields dense cells and ISC with rheological properties similar to those of endogenous dense sickle cells [24].

Reticulocytes were counted after supravital staining with new methylene blue. ISC were counted (percentage of cells with characteristic elongated, boat-shaped morphology [24]) using interference microscopy after fixation of fully oxygenated cells in 1.25% v/v glutaraldehyde in HBS.

Endothelial Cell Culture

Endothelial cells were harvested from human umbilical vein and grown to confluence in 25-cm^2 flasks in Medium M199 (ICN Flow Laboratories, High Wycombe) supplemented with 4 mmol/L L-glutamine (Sigma Chemical Company, Poole), 50 IU/ml sodium heparin (CP Pharmaceuticals, Wrexham) and 20 % v/v normal human serum, as described [25]. Care was taken to ensure that the serum used was ABO blood group compatible with the endothelium. Once confluent, the cells were removed from the flask using EDTA/trypsin and were used to seed glass "microslides" (rectangular glass tubes, cross-section 300 × 3,000 μm, with good optical qualities; Camlab, Cambridge, UK) using the method previously described by Cooke et al. [25]. The cells were grown to confluence for 24 hr before use in the flow adhesion assay.

Flow Adhesion Assay

A microslide containing confluent endothelial cells was glued to a glass microscope slide and placed on a light microscope stage so that the endothelial surface could be viewed. Using double-sided adhesive tape, silicon rubber tubing was attached to each end of the microslide and connected at one end to a Harvard infusion/withdrawal syringe pump and at the other to an electronic 3-way microvalve, which allowed the flow of either red cell suspension or wash solution over the endothelium [25]. All experiments were carried out at room temperature (23°C) except in limited later studies when the assay system was enclosed in a thermostatically controlled box at 37°C.

The red cell suspension (0.5×10^{12} red cells/l in M199 with 10% v/v autologous plasma added unless stated otherwise; pH 7.4) was flowed over the endothelium for 5 min, followed by cell-free suspending medium at the same flow rate to wash out nonadherent cells. For each sample, adherent cells were counted in a number of complete microscope fields of known dimensions, averaged per field, and then expressed as cells/$mm^2/10^8$ cells perfused, which takes into account variation in flow rate between assays (i.e., a measure of efficiency of adhesion). The effect of wall shear stress imposed by flow was studied by performing the adhesion assay at a wall shear stress of 0.02. 0.05 or 0.1 Pascals (Pa). For subsequent assays, wall shear stress was 0.05 Pa. Wall shear stress was calculated from the controlled flow rate, microslide dimensions, and the viscosity of the suspending medium (i.e., 10% plasma in M199, assuming negligible contribution to viscosity from suspended red cells). The effect of plasma concentration was assessed by including autologous plasma in the suspension medium at concentrations within the range 0–100% v/v. In these experiments, either (1) the wall shear rate was held constant, so that the shear stress increased as plasma concentration was increased; or (2) the wall shear stress was held constant, so that shear rate was reduced as plasma concentration was increased; the shear rate was adjusted between samples according to the variation in the viscosity of the medium, which was measured by a plasma viscometer (Coulter Viscometer II, Coulter Electronics).

Comparisons between treatments or cell fractions were made by measuring adhesion/$mm^2/10^8$ cells perfused (or supporting hematological parameters) for each fraction from a series of different donors and by calculating mean values and standard error of the mean (SEM) for the donors. Statistical comparisons between treatments or cell fractions were made using Wilcoxon's signed-rank test (two-tailed) for paired data derived from the individual donors.

RESULTS

Variation of Assay Conditions

We investigated experimental conditions potentially influencing differential adhesion of sickle cell fractions. The effect of varying the concentration of autologous plasma in the suspending medium is shown in Figure 1. The number of adherent cells tended to increase up to a concentration of 10% v/v plasma and then decreased again at higher concentrations. In these experiments, the wall shear rate was constant ($\sim 50 s^{-1}$), so the shear stress increased with the plasma concentration (stress was calibrated at 0.05 Pa at 10% v/v plasma). When human albumin was added instead of plasma, it had no consistent effect on adhesion at concentrations up to 30 g/L (Fig. 1). In separate experiments, we compared adhesion in 10% and 100% v/v plasma at constant shear stress (i.e., lower shear rate for the higher concentration), and found that the lower concentration supported slightly greater adhesion (163 vs. 129 cells/$mm^2/10^8$ cells perfused; mean of three experiments). In these studies of plasma proteins, and indeed elsewhere in the study, there was great interdonor variation in the level of adhesion. Although the presence of 10% v/v plasma did increase adhesion slightly in each experiment (compared to no plasma), there was no significant difference between concentrations. All subsequent experiments were carried out in the presence of 10% v/v autologous plasma, which appears to yield maximal adhesion.

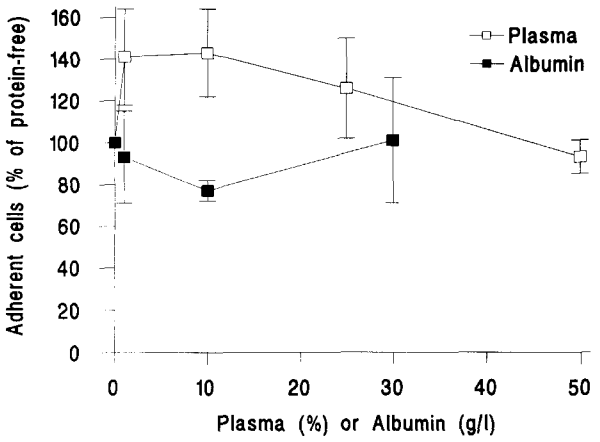

Fig. 1. Effect of concentration of plasma or of human serum albumin added to suspending medium on adhesion of flowing, unfractionated sickle cells to cultured endothelial cells. Wall shear shear rate was 50 s^{-1} and shear stress was 0.05 Pa for 10% v/v plasma. The same shear rate was applied for all plasma concentrations, and so the shear stress increased with increasing plasma concentration and suspending medium viscosity. A similar but lesser effect applies for albumin, which has a lesser influence on viscosity. Data are mean ±SEM from four experiments with plasma, and mean ± range from two experiments with albumin.

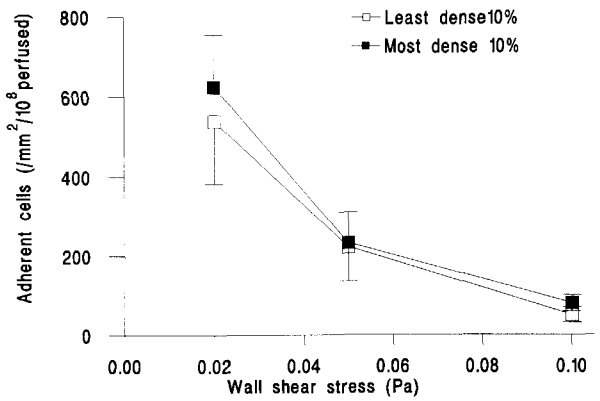

Fig. 2. Effect of concentration of wall shear stress on adhesion of flowing sickle cells to cultured endothelial cells. The least dense 10% (□) and most dense 10% (■) of cells separated by Percoll-Isopaque density gradient are compared. Data are mean ±SEM from seven experiments.

To assess the effect of wall shear stress, we measured adhesion of the least and most dense 10% of sickle cells (separated on a Percoll-Isopaque gradient) at 0.02, 0.05, and 0.1 Pa (Fig. 2). The level of adhesion decreased with increasing shear stress. Not only was the trend similar for the two fractions, but they had similar absolute levels of adhesion (Fig. 2). Subsequent studies were carried out at a wall shear stress of 0.05 Pa, because adhesion at 0.1 Pa was very low for some donors, while flow at 0.02 Pa was so slow that washout of nonadherent cells took long periods. At 0.05 Pa, we also tested the adhesiveness of unfractionated red cells from three HbAA donors in 10% autologous plasma and found levels of adhesion that were consistently low ($<5/mm^2/10^8$ cells perfused) and less than adhesion for any HbSS donor. Since we and others previously demonstrated that flowing HbSS red cells are more adhesive to endothelial cells than are HbAA red cells [8,9,18], this aspect of the study was not pursued further.

Comparison of Different Density Fractionation Procedures

We investigated the properties of different density fractions further. Sickle cells were separated either by density gradient or Wintrobe tube centrifugation; in either case, the least dense 10% fraction showed a high MCV and was rich in reticulocytes and contained few ISC (Table I). The most dense fraction showed a low MCV, low reticulocyte count, and high proportion of ISC. When comparing the two separation methods, there was no significant difference in the MCV, reticulocyte, and ISC counts for the least dense fractions. The most dense fraction showed a significantly lower MCV using the density gradient, suggesting a slightly better resolution of dense cells (Table I). However, the difference in reticulocyte and ISC were not significant between the two different types of dense fraction.

Using density-gradient separation, adhesion to endothelium was significantly higher in the least and most dense fractions compared to the middle fraction (Fig. 3). However, when using Wintrobe tube separation, although the adhesion of the light fraction was elevated, adhesion in the dense 10% fraction was not higher than the middle fraction (Fig. 3). We performed further experiments to investigate this discrepancy.

Using the Wintrobe tube centrifugation method, we had noted significant hemolysis when washing the most dense fraction, presumably caused by packing of cells against the glass tube at the bottom of the column. We found that hemolysis could be prevented by inserting a layer of dense Percoll/Isopaque mixture at the bottom of the Wintrobe tube, which then acted as a cushion for the cells as they were centrifuged. In three experiments using this technique, however, we found that the results were similar to those previously obtained (i.e., the dense fraction showed adhesion approximately equal to the middle fraction, but the top fraction was more adherent; adhesion ratio of dense/middle = 1.0 ± 0.1 and of top/middle = 6.0 ± 2.9; mean ± SEM from three experiments). Therefore, the low adhesion of the dense fraction separated using the Wintrobe technique could not be attributed to excessive hemolysis of highly adhesive cells.

We tested whether exposure to the density gradient or its components led to an increase in the adhesive properties of sickle cells. We incubated cells in isotonic solutions

TABLE I. Hematological Properties of Sickle Cells Fractionated by Density Gradient or by Centrifugation of Packed Cells†

	Density gradient	Centrifugation
Proportion of cells (%)[a]		
Least dense	15.8 ± 1.7	10
Middle	74.0 ± 2.2	80
Most dense	10.2 ± 1.0	10
Mean cell volume		
Least dense	101.7 ± 4.5	103.9 ± 5.0
Middle	86.6 ± 3.1	90.5 ± 4.3
Most dense	67.9 ± 3.9*	73.8 ± 4.7*
Reticulocytes (%)		
Least dense	14.2 ± 1.8	20.1 ± 3.7
Middle	3.8 ± 0.9	3.3 ± 0.4
Most dense	1.4 ± 0.5	1.3 ± 0.4
Irreversibly sickled cells (%)		
Least dense	6.5 ± 2.3	8.5 ± 2.8
Middle	13.0 ± 3.2	13.9 ± 3.9
Most dense	64.8 ± 6.9	56.2 ± 9.3

†Data are mean ±SEM from six experiments.
[a]Proportion of cells was based on counting for density gradient but on cutting of the Wintrobe tube for packed cells.
*$P < 0.05$ for comparison between separation methods.

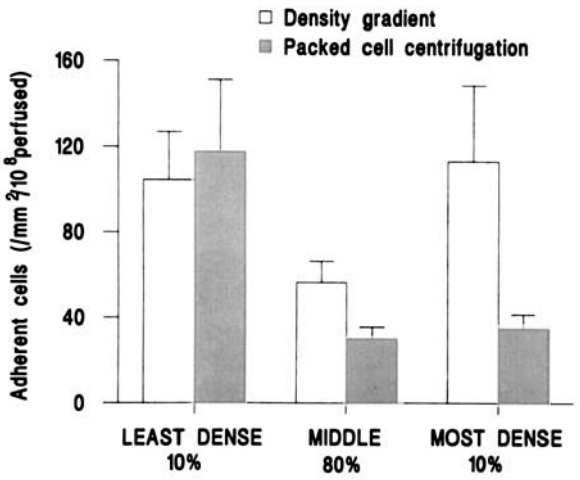

Fig. 3. Adhesion of different density fractions of sickle cells to cultured endothelial cells. The least dense 10%, most dense 10%, and middle 80% of cells are compared for two different separation procedures: Percoll-Isopaque density gradient (□) and packed cell centrifugation (■). Data are mean ±SEM from 11 experiments. Comparing the gradient to the packed cell method, the gradient gave higher adhesion for the middle and most dense cells ($P < 0.01$ for both) but not the least dense cells. Comparing fractions, the least dense cells were more adhesive than the middle for either separation technique ($P < 0.01$ in each case), but the most dense cells were more adhesive than the middle only for the gradient technique ($P < 0.01$).

of Percoll and Isopaque for 40 min; after washing three times, we compared the adhesion of these cells with cells that had been incubated in buffer alone and with cells that had been subjected to density-gradient centrifugation and recombined. Exposure to Isopaque caused no change in adhesiveness (Table II). Percoll tended to cause a reduction in adhesion, while exposure to gradient centrifugation tended to increase adhesion, but neither effect was consistent in studies of four different donors (Table II). Next, the dense fraction produced by density gradient was centrifuged in Wintrobe tubes, to see whether centrifugation itself would produce a loss of adhesion. In two such experiments, we found that the adhesion was markedly reduced after the cells had undergone the Wintrobe centrifugation (mean adhesion fell from 80 to 24 cells/mm^2/10^8 perfused). Conversely, we prepared the dense fraction by Wintrobe centrifugation and subjected these cells to the density-gradient procedure. In two experiments, the density gradient caused no change in adhesion (mean adhesion was 55 and 57 cells/mm^2/10^8 perfused, before and after gradient, respectively).

Effect of In Vitro Incubation and Formation of Dense Cells

We previously showed that when less dense sickle cells were subjected to 15 hr of cyclical deoxygenation–reoxygenation in the presence of calcium, they lost potassium and water via the Gardos channel and a proportion became ISC; 15-hr incubation without deoxygenation did not affect these parameters [26]. Here, cyclically deoxygenated cells showed significantly higher adhesion than control cells incubated at 37°C but kept fully oxgenated (Table III). However, it was notable that both sets of cells incubated at 37°C showed lower adhesion than cells kept fully oxygenated at 4°C (Table III). At 37°C, cell maturation occurred (indicated by a reduction in reticulocyte

TABLE II. Effect of Exposure to Density Gradient and Its Components on Adhesion of Flowing Sickle Cells to Cultured Endothelial Cells*

	Adhesion (cells/mm²/10⁸ perfused)				
	Patient				
Treatment	1	2	3	4	Mean ±SEM
Control (buffer alone)	108	108	76	19	78 ± 21
Incubation in Percoll	97	74	34	24	57 ± 17
Incubation in Isopaque	103	98	97	25	81 ± 18
Density gradient and recombined	92	95	203	73	116 ± 29

*Data are from four different patients tested once each. Wall shear stress was 0.05Pa in all assays.

TABLE III. Effect of Incubation With and Without Cyclical Deoxygenation on Maturation of Sickle Cells and Adhesion to Cultured Endothelial Cells†

Incubation	Reticulocytes (%)	Irreversibly sickled cells	Adherent cells (/mm²/10⁸ perfused)
15 hr at 4°C	8.7 ± 1.1	6.7 ± 1.6	38.7 ± 10.4
15 hr at 37°C	5.4 ± 0.7*	5.4 ± 0.8	8.3 ± 2.3*
15 hr at 37°C with cyclical deoxygenation	2.8 ± 0.6**	55.1 ± 7.8**	16.3 ± 5.2**

†Data are mean ±SEM from nine experiments. Wall shear stress was 0.05 Pa in all adhesion assays.
*$P < 0.05$ for comparison between 15 hr incubation at 37°C and 4°C.
**$P < 0.05$ for comparison between 15 hr incubation at 37°C with or without cyclical deoxygenation.

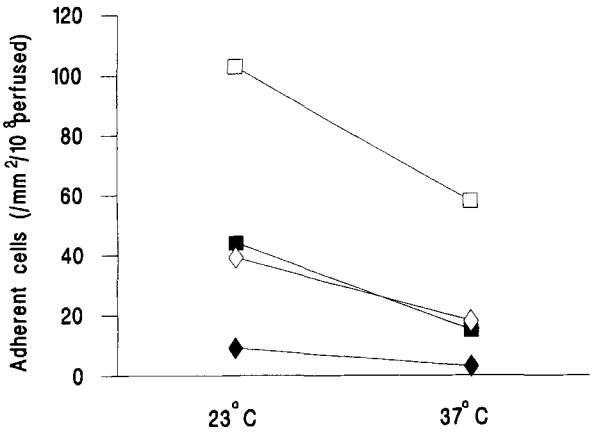

Fig. 4. Effect of temperature on adhesion of flowing, unfractionated sickle cells to cultured endothelial cells. Wall shear stress was 0.05 Pa for both 23°C and 37°C, and so the shear rate was about 40% higher at the higher temperature (to compensate for reduced suspending medium viscosity). Data are shown individually for four paired experiments.

count), while ISC were formed only after cyclical deoxygenation at 37°C (Table III).

All adhesion measurements were at room temperature (23 ± 2°C), because the apparatus to carry out assays at 37°C was only developed late in the study. Evaluating the effect of temperature for unfractionated cells, we found that adhesion was consistently reduced at 37°C, about one-half the level at 23°C on average (Fig. 4). Shear stress was the same for the two temperatures (0.05 Pa), so the shear rate was about 40% higher at 37°C to compensate for the lower medium viscosity.

DISCUSSION

Adhesion of sickle cells to vascular endothelium could play a role in promoting vascular occlusion, either mechanically or by delaying cell transit and increasing the likelihood of sickling in the microvasculature. The mechanisms for this adhesion is probably multifactorial and has been shown to be influenced by erythrocytic factors such as morphology, dehydration, and membrane mechanics [15,20], by increased binding of IgG [27], and by expression of antigens particular by immature reticulocytes [14,16,17], as well as by plasma factors such as vWF, fibronectin [18], thrombospondin [12], and autologous platelets [7]. The evidence for which cells among the heterogeneous population in sickle cell disease are most adhesive has nevertheless been contradictory. A number of studies agree that young cells adhere preferentially [8,11,14], but the relative adhesion of dense cells has varied among studies [6,8,11,14,15]. Dense cells adhered more than unfractionated cells after stimulation of endothelial cells with tumor necrosis factor (TNF) for 4 hr [19]. After 6-hr stimulation with the same cytokine, adhesion of presumably young sickle cells bearing the $\alpha_4\beta_1$-integrin was increased via the endothelial receptor VCAM-1 [17]. We set out to reconcile these apparent differences and found that not only were less dense cells more adhesive than the main population, but that dense and dehydrated

cells were also unusually adhesive. However, the results were dependent on the method of sample preparation.

Using a Percoll-Isopaque continuous density gradient we were able to demonstrate high adhesion of the 10% least dense reticulocyte-rich fraction as well as high adhesion of the 10% most dense, ISC-rich fraction, as compared to the middle 80%. Many adherent cells among the least dense fraction had a morphological resemblance to stress reticulocytes [28], so that our results support the concept that young sickle cells are particularly adhesive and that initial stages of maturation are associated with a decrease in adhesiveness. The dense cell fraction contained about 10 times less reticulocytes than the least dense fraction but adhered approximately equally, suggesting that a process of dehydration and membrane damage in vivo might underlay renewed adhesive potential after initial maturation. However, there is evidence that some dense sickle cells may be relatively young [29] and, if these cells retained adhesion receptors characteristic of reticulocytes [14,16,17], this could also be a factor in the retention of abnormal adhesiveness.

We obtained further information from studies of sickle cells incubated in vitro. The less-dense 50% of cells had reduced reticulocyte count and adhesiveness after incubation at 37°C for 15 hr compared to identical cells held at 4°C, suggesting that maturation, even in vitro, may be associated with rapid loss of adhesiveness. There is a caveat, in that cells held at 4°C tended to have lower adhesion compared to cells which were tested fresh in other, unpaired experiments (e.g., Fig. 1, Table II), so that adhesive properties may deteriorate under any form of storage. Nevertheless, cells subjected to 15 hr of continuous cycles of deoxygenation–reoxygenation at 37°C showed significantly higher adhesion than incubated control cells kept fully oxygenated. There was greater loss of reticulocytes during the cycles, and many adherent, cyclically deoxygenated cells resembled ISC. Oxidative stress and dehydration have previously been linked to increased adhesiveness of red cells [5] and could also underly the pro-adhesive effects of repeated sickling. Similar processes may contribute to the increased adhesiveness of endogenous dense cells, since the cyclical deoxygenation model has been found previously to reproduce other rheological abnormalities found in naturally occurring dense cells [24]. Again, a caveat should be added because of the unknown status of adhesion receptors such as CD36 and VLA-4 on endogenous and artificially produced dense cells; the literature associates these receptors with reticulocytes but these reports do not give their distribution among different density fractions [14,16,17].

When density fractions were prepared by centrifugation of packed cells in Wintrobe tubes, different results were obtained. The least dense 10% fraction again showed high adhesion, but the adhesion of the most dense 10% fraction was similar to the middle 80%. The two separation procedures yielded dense cells with similar hematological properties, although the MCV was somewhat lower for the gradient method, which may indicate a slightly more efficient separation. However, in view of the large difference in adhesion between the different dense fractions, which represented similar proportions of the red cell population, it seems likely that either the Wintrobe tube method caused some damage to the dense cells, which decreased their adhesive properties, or that the components of the density gradient somehow promoted adhesion of dense cells. We were unable to show that the Percoll-Isopaque gradient or any of its components had any consistent effect on adhesion. Hemolysis was evident in the dense fraction from the Wintrobe tube method, but alleviation of lysis using a dense cushion did not alter adhesion results. Nevertheless, we did note that dense cells from the Percoll-Isopaque gradient became less adhesive if subsequently centrifuged in Wintrobe tubes. We think it most likely that during centrifugation in Wintrobe tubes, tight packing of the dense cells in the glass tube results in membrane damage and loss of adhesiveness for endothelial cells. The results from the two types of separation used here are consistent with, and explain discrepancy between, previous studies. Attention to the detail of methods used by others shows that those studies in which dense cells were more adhesive than average used isopycnic density gradients [6,11,19], whereas those in which dense cells were not more adhesive used centrifugation of packed cells [8,14]. In the light of these results, we also suggest that the use of an isopyknic density gradient is the method of choice for fractionation of sickle cells, avoiding hemolysis induced during centrifugal packing.

Comparisons between subpopulations were all made in the presence of shear flow, albeit at shear stress at the low end of the physiological range [30]. Plasma was also present in the adhesion assay medium and tended to increase adhesion, although the effect was not great, and the percentage of plasma was not critical. Others have found that plasma from sickle patients has a greater effect on adhesion to microvascular endothelial cells compared to umbilical vein endothelial cells [21], so this factor could be more important in vivo. Our comparison of fractions was made at room temperature. We did note that adhesion of unfractionated cells was consistently lower at 37°C, probably because the flow rate was 40% higher at the higher temperature (to compensate for lower medium viscosity in applying equal shear stress). Increased flow rate usually occurs with parallel increase in shear stress, and it is evident that when this occurs, adhesion becomes less efficient (Fig. 2). Considering these factors separately, adhesive bonds must have rapid forward kinetics to operate in flow [31] and the initial bond formation is probably predominantly dependent on time of contact and

hence flow rate. Upon formation of a bond, shear stress on the cell translates into a force acting on the bond, which tends to break or reverse it. While increasing flow rate at constant stress, or vice-versa, would both be predicted to reduce efficiency of stable attachment, it is hard to judge which is the dominant factor in controlling adhesion in the current system. Since kinetic effects apply to all cells at all temperatures, comparison of fractions made at room temperature should also apply in the circulation. Interestingly, others have noted increased adhesion of sickle cells at 37°C compared to room temperature [7], but this was in static assay, where kinetics do not limit adhesion. It seems that the equilibrium adhesive state is enhanced at higher temperature.

The physiological implications of this study are that dense sickle cells as well as reticulocytes might adhere to vascular endothelium in vivo. Dense cells already have an increased tendency to sickle because of their higher hemoglobin concentration, and any delay in their transit through the venous circulation could prove catastrophic. Animal studies have suggested that less dense cells adhere in venules, while dense cells tend to become trapped earlier in the arteriolar and capillary network, presumably via a mechanical rather than adhesive mechanism [12,13,32]. A recent investigation perfusing sickle cells through ex vivo rat mesocecum showed that although dense sickle cells induced marked resistance to flow that was not fully recoverable, they were less adhesive in venules than the least dense fraction [15]. Moreover, dehydration of the latter cells via selective changes in membrane permeability impaired their adhesiveness. These results seem to be at odds with those presented here, where comparable levels of adhesion were seen for least and most dense sickle cells and cyclical deoxygenation induced both dehydration and increased adhesion. One possibility is that trapping of dense cells by mechanical means reduces their availability for adhesion in the ex vivo model. Marked trapping and blockage of microvascular beds in the human may not be continuous in the steady state, perhaps because of adaptive or damage-induced changes in the microvascular architecture. It could thus be that both mechanical and adhesive abnormalities of dense cells, along with adhesion of HbSS reticulocytes, all contribute to the switch from steady state to occlusive crisis, driven by as yet uncertain precipitating factors.

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